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This report will provide a final summary of project accomplishments, emphasizing those of approximately the past year of the project. Annual reports previously submitted to AFOSR are incorporated herein by reference. Most recently, we used DNA microarray analyses to characterize stress responses of wild-type Synechocystis sp. PCC 6803 cells encapsulated in different gel formulations. We also characterized photosynthetic activity, encapsulation protocols, and hydrogen production from photoantennae mutants of Synechocystis sp. PCC 6803.In addition, we continued exploring the diffusion of dissolved ions encapsulated in silica gel.

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Final Report

Project Title: Engineered, Solid-State Processes for Enhanced Biosolar Hydrogen Production and Exploitation of Solar Energy with Tailored Light-Harvesting Systems

AFOSR Grant Number: FA9550-08-1-0448

Investigators: PI: Roger Ely, Oregon State University; co-PI: Catherine Page, University of Oregon; co-PI: David Kehoe, Indiana University

Summary of Accomplishments

Introduction. This report will provide a final summary of project accomplishments, emphasizing those of approximately the past year of the project. Annual reports previously submitted to AFOSR are incorporated herein by reference. Most recently, we used DNA microarray analyses to characterize stress responses of wild-type *Synechocystis* sp. PCC 6803 cells encapsulated in different gel formulations. We also characterized photosynthetic activity, encapsulation protocols, and hydrogen production from photoantennae mutants of *Synechocystis* sp. PCC 6803.In addition, we continued exploring the diffusion of dissolved ions encapsulated in silica gel.

Microarray Analyses of Encapsulated Cells. Microarray analyses were performed on cells encapsulated in gels derived from aqueous precursors and gels derived from alkoxide precursors and incubated under constant light for 24 hours prior to RNA extraction. For comparison purposes, cultures suspended in liquid media were also exposed to 500 mM salt stress and incubated under identical conditions. The expression of 414 genes was significantly altered by encapsulation in aqueous-derived gels (fold change ≥ 1.5 and P-value < 0.01), the expression of 1143 genes was significantly altered by encapsulation in alkoxide derived gels, and only 243 genes were common to both encapsulation chemistries (see Table I; for additional details regarding changes in gene expression, see publication described below). Additional qRT-PCR analyses of four select genes; *ggpS*, *cpcG2*, *slr5055*, and *sll5057*, confirmed microarray results. These results illustrate that encapsulation presents a unique type of stress that warrants further investigation.

Among the 20 most strongly up- and down- regulated genes, nine genes were common to both encapsulation chemistries and regulated in the same direction. Up-regulated genes included slr1291 (NADH dehydrogenase subunit 4, NdhD2) and sll1471 (phycobilisome rod-core linker polypeptide CpcG2). Down-regulated genes included slr0772 (light-independent protochlorophyllide reductase subunit ChlB), and six hypothetical proteins: slr1667, slr1152, slr0888, sll0382, slr1957, and sll0381. Of the statistically significant differentially regulated encapsulation and alcohol stress genes identified, approximately half were for hypothetical and unknown proteins, suggesting that much remains to be learned about encapsulation stress responses, and with possible implications for improving encapsulation protocols and rationally engineering microorganisms for direct biofuel production. (Publication: Dickson, DJ, Luterra, MD, and RL Ely. 2012. Transcriptomic Reponses of *Synechocystis* sp. PCC 6803 Encapsulated in Silica Gel. Appl Microbiol Biotechnol. **96**(1):183-196)

Viability and Photosynthetic Activity of Encapsulated Cells. Chlorophyll fluorescence was used to explore the response of wild-type *Synechocystis* sp. PCC 6803 to short term (2 minute) exposure to, and 24-hour recovery from, common compounds present in biological encapsulation which may stress the organism or protect from stress, including salt, ethanol, glycerol, PEG200, and betaine. It was found that *Synechocystis* sp. PCC 6803 is highly sensitive to an initial stress presented by exposure to all these compounds after two minutes of exposure (Figure 1). After 24 hours, surprisingly, the cells recovered

Table 1. Differentially regulated genes by ≥1.5-fold and *P* value<0.01 in response to encapsulation in aqueous-derived gels, and exposure to 500 mM NaCl in liquid culture, compared to controls in BG-11 media.

Functional Category	Number of genes	Differentially expressed genes – aqueous encapsulation	Differentially expressed genes – alkoxide encapsulation	Affected genes common to both encapsulation chemistries *	Differentially expressed genes – 500mM NaCl Treatment
Amino Acid Biosynthesis	97	9	29	3 (3)	59
Biosynthesis of cofactors, prosthetic groups, and carriers	125	16	33	9 (0)	75
Cell Envelope	67	6	21	5 (0)	34
Cellular Processes	78	15	25	10 (2)	50
Central Intermediary Metabolism	31	7	11	5 (2)	21
DNA replication, restriction, modification, recombination, and repair	74	1	19	0 (0)	36
Energy Metabolism	93	11	23	3 (0)	52
Fatty acid, phospholipid and sterol metabolism	39	7	15	5 (1)	24
Hypothetical	1225	138	361	76 (17)	689
Other Categories	363	20	117	10 (6)	162
Photosynthesis & Respiration	143	27	58	15 (2)	112
Purines, pyrimidines, nucleosides, and nucleotides	43	3	14	2 (0)	24
Regulatory Functions	156	8	53	5 (2)	88
Transcription	30	5	18	4 (1)	24
Translation	168	14	63	12 (0)	90
Transport & Binding	200	42	83	21 (9)	99
Unknown	635	85	200	49 (12)	297
Total	3567	414	1143	234 (57)	1936

* Numbers in parentheses indicate the number of genes affected by both encapsulation treatments, but regulated in opposite directions. For example, of the 10 genes of the "cellular processes" functional category, 8 were regulated in the same direction by both encapsulation treatments, while 2 were regulated in opposite directions.

from ethanol and salt stress, as indicated by a recovery in quantum efficiency, but did not recover from exposure to the other compounds (Figure 2). We have shown that glycerol, used widely in encapsulation of other organisms to serve as a protectant, is detrimental to *Synechocystis* sp. PCC 6803 (and likely other cyanobacteria) because it interferes with excitation transfer between photoantennae and photosystems. Polyethylene glycol, another common additive, was similarly detrimental, which is extremely important information for creating biocomposites via bio-encapsulation.

A similar approach was used to examine responses to encapsulation, with and without the above additives. It was determined that glycerol and PEG200 were detrimental to long term viability, while betaine had little impact. Figure 3 illustrates stable quantum efficiency of PSII in both alkoxide and aqueous gels, with no additives, over a period of six weeks (Publication: Dickson, DJ and RL Ely. 2011. Evaluation of Encapsulation Stress and the Effect of Additives on Viability and Photosynthetic Activity of *Synechocystis* sp PCC 6803 Encapsulated in Silica Gel. Appl Microbiol Biotechnol. **91**(6):1633-1646). Importantly, our results also showed that diffusion through the silica gels is adequate to support viable cells of *Synechocystis* sp. PCC 6803 for six weeks or more. The ultimate longevity of encapsulated *Synechocystis* sp. PCC 6803 cells is not yet known.

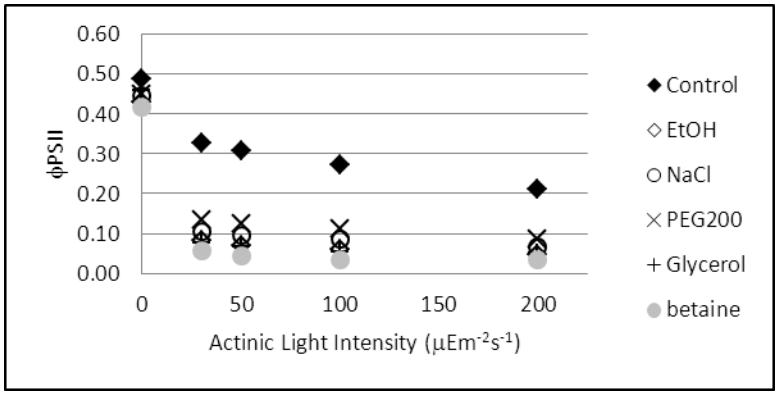

Figure 1 - Responses of *Synechocystis* sp. PCC 6803 to two-minute exposure to indicated compounds.

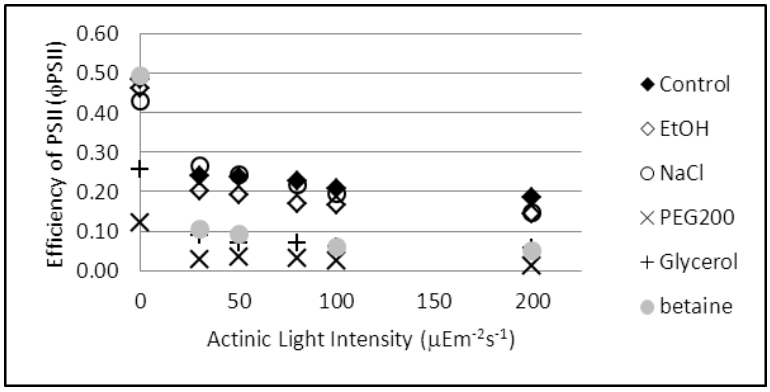

Figure 2 – 24-hr recovery of *Synechocystis* sp. PCC 6803 cells after exposure to indicated compounds.

Hydrogen Production from Photoantennae Mutants. Cultures of three photoantennae mutants of *Synechocystis* sp. PCC 6803, named ΔcpcAB, ΔapcE, and Δapcf, were conditioned in nitrogen- and sulfur-starved media to assess glycogen accumulation, which is required for fermentative hydrogen production. Under 200 $\mu Em^{-2}s^{-1}$ light, all strains accumulated similar levels of glycogen (not shown). Under 400 $\mu Em^{-2}s^{-1}$ light, the ΔcpcAB strain accumulated 30% less glycogen than other strains, as shown in Figure 4.

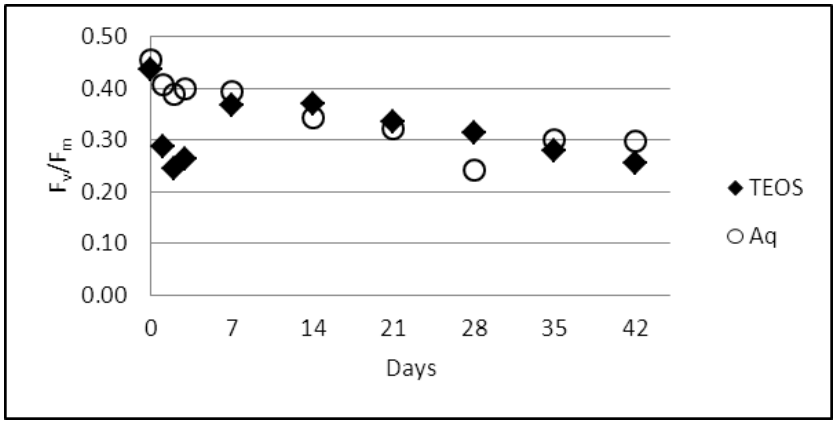

Figure 3 - Quantum efficiency of *Synechocystis* sp. PCC 6803 cells not exposed to additives.

Encapsulated cells conditioned with light at 200 $\mu Em^{-2}s^{-1}$ and incubated anaerobically showed similar hydrogen production from all strains (not shown). Conditioned under 400 $\mu Em^{-2}s^{-1}$ light, hydrogen production from the ΔapcE and ΔapcF strains was 25% higher than from the WT, reaching a headspace concentration of 1.2%. (ΔcpcAB produced less hydrogen than WT). Also, hydrogen production from cells encapsulated in silica gels, especially aqueous-derived gels, was typically 2.5 to 3.5 times higher than from comparable liquid controls (Figure 5). Also, Figure 6 shows that ΔcpcAB cells were much more efficient than other strains, especially wild-type cells, at producing hydrogen, based on the amounts of pigments they contained. Our results verify that manipulation of light harvesting antennae and silica sol gel encapsulation can complement each other to yield greatly enhanced photobiological hydrogen production (manuscript submitted to International Journal of Hydrogen Energy, currently in revision).

We chose to examine photoantennae mutants because it is known that phototrophs with truncated photoantennae become saturated at higher light intensities compared to wild-type organisms. This means that, for a culture as a whole, they may use incident light more efficiently. Additionally, to our knowledge, this is the first investigation to demonstrate improved hydrogen production from such a mutant. Furthermore,

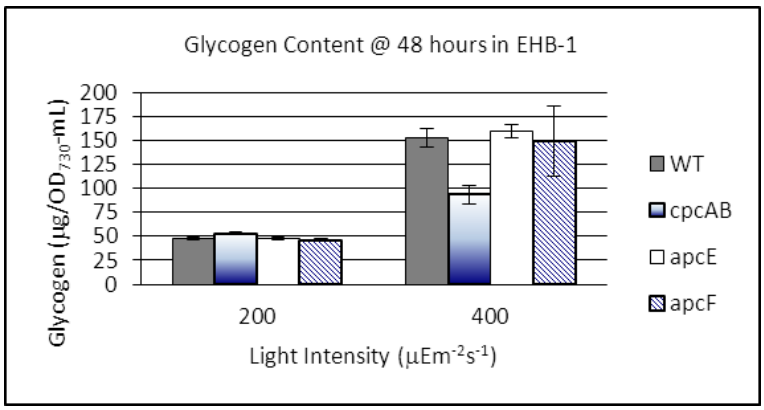

Figure 4 - Glycogen content of wild type and antenna mutant strains of *Synechocystis* sp. PCC 6803 cells exposed to 200 and 400 $\mu Em^{-2}s^{-1}$ of light.

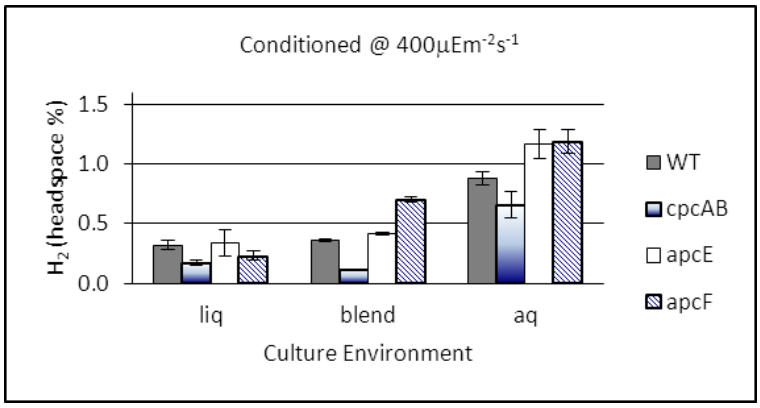

Figure 5 - Hydrogen production from *Synechocystis* sp. PCC 6803 cells incubated anaerobically after conditioning at 400 $\mu Em^{-2}s^{-1}$.

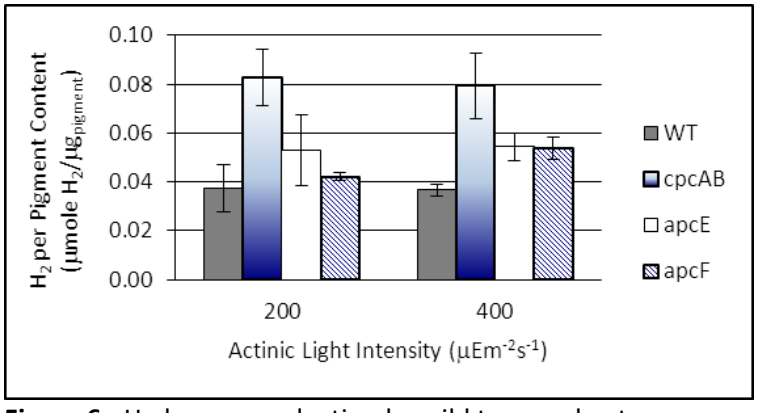

Figure 6 - Hydrogen production by wild type and antenna mutants of Synechocystis sp. PCC 6803, normalized to the amount of pigment content.

we have refined encapsulation protocols to improve encapsulation in gels derived from aqueous precursors, which we have shown to be advantageous for hydrogen production compared to alkoxide gels. (At this time, it is not yet known which formulation will be better for maintaining long-term maximal viability and activity.) Future work could continue to characterize photosynthetic activity of these antennae mutants to further improve the efficiency of the encapsulated system toward long term viability and hydrogen production.

Our research has demonstrated that antennae mutants can produce hydrogen at rates comparable to or higher than those of WT controls, storing comparable levels of glycogen with less photopigment content, as shown in Figure 4. Also, we have confirmed our initial hypothesis that, done properly, encapsulation can significantly enhance hydrogen production. Future work could continue improving protocols, exploring higher light intensities, and using tailored silica encapsulation approaches with antennae mutants to increase light utilization efficiency and hydrogen production.

Diffusion Properties of Gels. We used divalent nickel (Ni^{2+}), Cu(II)EDTA, methyl orange, and dichromate to investigate diffusion from hydrated silica sol–gel monoliths. The objective was to examine diffusion of compounds on a size regime relevant to supporting biological components encapsulated within silica gel prepared in a biologically compatible process space with no post-gelation treatments. In work done earlier in the project with an initial sample set, we used a factorial experimental design to explore gels prepared from tetraethoxysilane with Ni^{2+} as the tracer, varying water content during hydrolysis, acid catalyst present during hydrolysis, and the final concentration of silica. A second sample set explored diffusion of all four tracers in gels prepared with aqueous silica precursors and a variety of organically modified siloxanes. Excluding six outliers which displayed significant syneresis, the mean diffusion constant (D_{gel}) across the entire process space of sample set 1 was $2.42 \times 10^{-10} m^2 s^{-1}$; approximately 24% of the diffusion coefficient of Ni^{2+} in unconfined aqueous solution. In sample set 2, the tracer size and not gel surface chemistry was found to be the primary determinant of changes in diffusion rates. A strong linear inverse correlation was found between tracer size and the magnitude of D_{gel} (Figure 7). Based on correlation with the tracers used in this investigation, the characteristic 1-h diffusion distance for carbonate species relevant to supporting active phototrophic organisms was approximately 1.5 mm. These results support the notion that silica sol–gel formulations may be optimized for a given biological entity of interest with manageable impact to the diffusion of small ions and molecules. These results were also used to model the diffusion behavior of dissolved carbonate species, which will facilitate design optimization for encapsulated systems. Typical gel structure at different scales is shown in Figure 8. (Publication: Dickson, DJ, Lassetter, B, Glassy, B, Page, CJ, Yokochi,

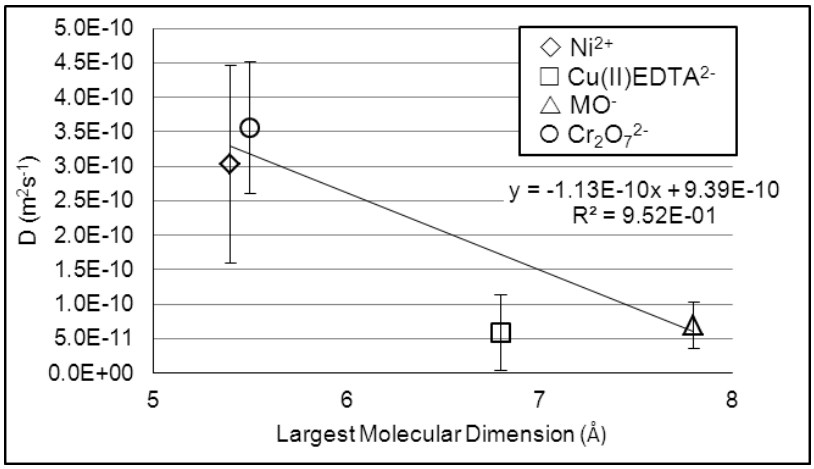

Figure 7 - Diffusion coefficient versus molecular size for diffusion of the indicated molecules in silica sol gel.

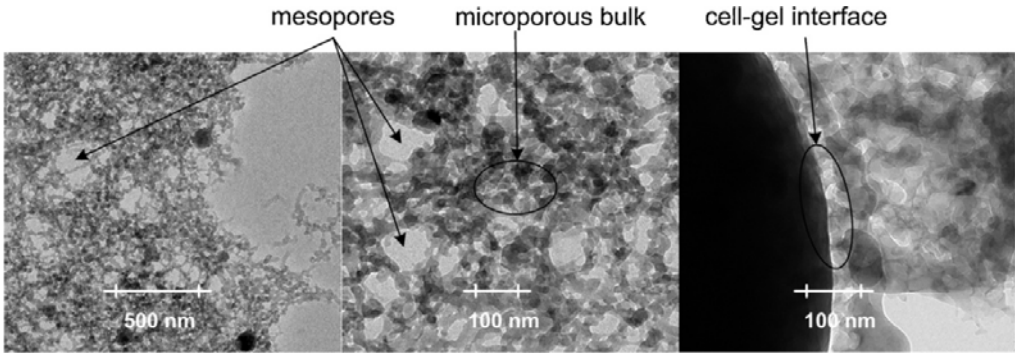

Figure 8 - TEM images of an alkoxide-derived gel containing cells of *Synechocystis* sp. PCC 6803.

AFT, and RL Ely. 2012. Diffusion of Dissolved Ions from Wet Silica Sol-gel Monoliths: Implications for Biological Encapsulation. Coll Surf B: Biointerfaces. In Press, published online 10 Sept 2012).

Overall Summary and Conclusion.

The total budget for this project was $931,873, which was originally to have been spread over a three-year period. Because of difficulties created when a key post-doc lost his legal status in the U.S., requiring that he be removed from the project, we requested a one-year, no-cost extension. Hence the project was conducted over a four-year period. Original project objectives, as stated in the 2008 proposal to AFOSR, were as follows:

(1) Determine gel composition and encapsulation procedure for optimal metabolic activity and stability of *Synechocystis* sp. PCC 6803 cells in sol-gel;
(2) Assess interactions between the sol-gel and the encapsulated cells to improve gel formulation and encapsulation technique and to assist future *a priori* design of encapsulation gels and approaches;
(3) Develop, characterize, and select modified strains of *Synechocystis* sp. PCC 6803 with tailored light harvesting antennae to harvest photosynthetically active radiation (PAR) more efficiently over a range of light intensities; and
(4) Characterize photosynthetic activity and hydrogen production from the encapsulated cultures to optimize gel geometry and optical properties for hydrogen production.

We are very gratified and proud that the original project objectives were accomplished completely and the project was a success. In addition, by conducting the DNA microarray studies within the constraints of the original budget, we were able to reach beyond the original objectives and begin to explore new questions related to encapsulation of living cyanobacterial cells.

Over the four-year period, the project supported a total of three post-doctoral researchers, two PhD students, and numerous undergraduate and high school student research assistants. So far, four papers have been published in respected journals and a fifth is currently in revision:

Dickson, DJ. and RL Ely. 2009. Photobiological hydrogen production from *Synechocystis* sp. PCC 6803 encapsulated in silica sol-gel. Int J Hyd Energy. **34**(1):204-215

Dickson, DJ and RL Ely. 2011. Evaluation of encapsulation stress and the effect of additives on viability and photosynthetic activity of *Synechocystis* sp. PCC 6803 encapsulated in silica gel. Appl Microbiol Biotechnol. **91**(6):1633-1646

Dickson, DJ, Luterra, MD and RL Ely. 2012. Transcriptomic responses of *Synechocystis* sp. PCC 6803 encapsulated in silica gel. Appl Microbiol. Biotechnol. **96**(1):183-196

Dickson, DJ, Lassetter, B, Glassy, B, Page, CJ, Yokochi, AFT, and RL Ely. 2012. Diffusion of dissolved ions from wet silica sol-gel monoliths: implications for biological encapsulation. Coll Surf B: Biointerfaces. In Press (published online 10 Sept 2012)

Dickson, DJ, Kehoe, DM, and RL Ely. Photobiological hydrogen production from three phycobilisome mutants of *Synechocystis* sp. PCC 6803 encapsulated in silica gel. Submitted to Int J Hyd Energy. Currently In Revision

In addition to the above publications, we were recently nominated by a professor at the University of Göttingen and invited by the editor in chief of the journal, Applied Microbiology and Biotechnology, to write a review paper entitled, "Encapsulation of Cyanobacteria: Lessons for Academic and Applied Research." Because of the support we received from the AFOSR and the work we have conducted in this project, we are now recognized internationally as leaders in silica sol gel encapsulation of cyanobacteria.

We very much appreciate the support of the AFOSR and the encouragement and support provided to us by Dr. Walt Kozumbo. We also acknowledge and appreciate Katie Wisecarver's wonderful assistance throughout the project. This project allowed us to make important progress to further the concept of direct, photobiological production of hydrogen gas from water. We strongly feel that the process offers great promise, and we are continuing to seek support for additional research and development.